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(54) Title: A METHOD FOR IMMOBILIZING A POLYPEPTIDE IN A POLYMER AND A MEMBRANE PRODUCED THEREBY		
(57) Abstract <p>The present invention relates generally to the immobilization or incorporation of polypeptides, especially enzymes or other bioactive polypeptides into polymeric matrixes, especially polyurethane, membranes produced by said polymers, and the utilization of such membranes in biosensors. A preferred type of biosensor is the needle sensor designed for <i>in vivo</i> monitoring of glucose which comprises a core platinum anode (2) coated with an insulating lacquer (3), the anode (2) is situated inside a stainless steel reference cathode (4) which is insulated from the anode (2) by a layer of epoxy resin (5). At one end, the tip, the electrode (1) has a detection surface (6), which is in an acute angle to the general direction of the electrode (1). At the other end, the base, the electrode (2) is provided with terminals (7) and (8) for the anode (2) and cathode (4), respectively. The terminals (7) and (8) are soldered to leads (9) and (10), respectively, which are connected to instrumentation used when performing measurements.</p>		

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A method for immobilizing a polypeptide in a polymer and a membrane produced thereby.

TECHNICAL FIELD

The present invention relates generally to the immobilization or incorporation of polypeptides, especially enzymes or other bioactive polypeptides into polymeric matrixes, membranes produced by said polymers, and the utilization of such membranes in biosensors or electro-chemical sensors.

BACKGROUND OF THE INVENTION

10 In the past enzymes have been utilized industrially as catalysts, particularly in the fermentation industry, and the like. In general, the enzymes were dissolved or dispersed in various aqueous media for promoting a chemical reaction. After completion of the reaction the enzyme is not
15 recovered, but discarded. However, in recent years enzyme immobilization techniques have been developed, which enable repeated or continuous use of enzymes in a stable and active immobilized state, whereby the areas of use for enzymes have been rapidly expanded, for example in the process industry,
20 and to analyses such as EIA (Enzyme Immuno Assay), and ELISA (Enzyme Linked Immuno Sorbent Assay).

The measurements of concentrations of various components in blood or other body fluids are very important for clinical diagnosis, and consequently a great number of improvements or developments in various kinds of quantitative
25 measurements have been achieved.

Among these achievements the development of enzyme sensors has received attention, and a number have been proposed which are able to effect rapid and continuous
30 measurements by employing membranes wherein enzymes have been immobilized.

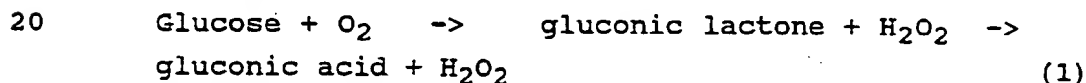
Information concerning the development of biosensors, their advantages and shortcomings may be found in the following review articles: "Biosensors, Fundamentals and
35 Applications" Eds. Turner, Karube and Wilson, Oxford University Press (1987) especially pages 409-424; Davis,

Biosensors, 2 (1986) 101-124 and Churchouse et al., Biosensors, 2 (1986) 325-342, which are hereby incorporated by reference.

Biosensors are typical examples of sensors utilizing
5 enzymes immobilized in membranes for the measurement of chemical substances. Such a biosensor comprises an enzyme immobilized in a membrane and a transducer adapted to detect substances consumed or produced in the membrane, which generates an electrical signal upon detection of such a
10 substance. In this case the enzyme immobilized in the membrane serves to discriminate a specific chemical substance to be measured, and cause a change in quantity of a material which corresponds to a change in the chemical substance and which is able to be detected by the
15 transducer.

Among such biosensors there are known those which employ glucose oxidase for the measurement of glucose.

Glucose oxidase acts to decompose glucose according to the following reaction:



Accordingly it is possible to measure the concentration (activity) of glucose by detecting the quantity of oxygen consumed, the quantity of hydrogen peroxide produced, or the
25 reduction in pH obtained in the above reaction inside the membrane.

In the enzyme sensors fabricated in the early years of this development, an enzyme immobilized in a membrane was physically or chemically applied to a sensitive portion of
30 an enzyme sensor which is adapted to convert physical or chemical quantities such as temperature, ion activity, gas activity or the like into electrical signals. Now, however, with miniaturization of enzyme sensors, it has become necessary to selectively form a membrane containing a
35 immobilized enzyme on the surface of a limited area of a sensitive portion of a sensor.

In order for these membranes to be functional in the biosensor in question they should fulfil a number of requirements depending on the type and nature of said biosensor.

5 Of such requirements a number may be mentioned, notably, stability in biological fluids, response over a clinically useful range, high selectivity, independence from variations in interfering substances, fast response, robustness, small size, stir independence, and
10 biocompatibility.

In order to fulfil such requirements sensors have been proposed which comprise multi-layer membranes of substantial complexity. In the sensors which have been proposed in the past, the immobilization has been achieved by chemically
15 binding the enzyme to the polymeric matrix. Such sensors are difficult and costly to produce in demanding considerable skill in the production, and the number of sensors that must be discarded is relatively large.

Specific examples of biosensors are described in the
20 following patents and patent applications.

US Patents Nos. 4,484,987 and 4,650,547 to Gough describe membranes useful in sensor devices, sensor devices, and the use of the membranes for determining a dissolved component in the presence of a gas reactive to said
25 component, such as glucose and oxygen in a solution.

German Patent Publication No. DE-A1-3335691 to Hitachi Ltd. discloses a urea electrode with a membrane comprising immobilized enzyme, which membrane is based on cross-linked albumin and treated with ethylenediamine in order to
30 introduce and increase the number of amino groups, whereby an increased permeability for ammonium ions is achieved.

In German Patent Publication No. DE-A1-2625544 a process for immobilizing biological material is disclosed, by which process the biological material is covalently bound to
35 free isocyanate groups in a polyurethane polymer through reactive amino groups in the biological material

BRIEF DESCRIPTION OF THE INVENTION

It has been an object of this invention to provide a simple and reliable method of immobilizing polypeptides, especially enzymes or other bioactive polypeptides into polymeric matrices by physical entrapment without covalently binding the polypeptide to the polymeric matrix, whereby the activity of the polymer could be decreased.

A further object of the present invention has been to provide for membranes produced by said polymeric matrices.

A still further object has been related to the utilization of such membranes in biosensors.

Yet another object of the invention has been to provide for a robust and reliable high quality biosensor incorporating membranes produced according to the method mentioned above.

The invention is described in further detail in the following detailed description and in connection with the appended examples and figures.

20

BRIEF DESCRIPTION OF THE DRAWING

In the drawings fig. 1 shows a longitudinal cross section of a single layer membrane needle electrode according to the invention,

fig. 2 shows an electrode according to the invention as used in a measuring set up, and

fig. 3 shows a cross section of a multi-layer membrane electrode of the invention corresponding to the longitudinal cross section of a single layer membrane needle electrode in fig. 1.

DETAILED DESCRIPTION OF THE INVENTION

As indicated above the invention in one of its aspects relates to a method for immobilizing a polypeptide such as

an enzyme in a polymeric matrix comprising the following steps:

- a) adding said polypeptide and a permeability modifying agent to an aqueous dispersion of a polymer to produce an aqueous dispersion wherein said polypeptide is dissolved;
- b) forming said dispersion into one or more bodies of a desired shape;
- c) leaving said shaped body for a period of time from about 5 minutes to about 200 minutes at room temperature for drying.

According to the invention a simple and reliable method of immobilizing a polypeptide, such as an enzyme, by which method only one mixing step is necessary between the active component and an inert aqueous dispersion of a polymer, and without any chemical or physical after treatment.

The active component thus retains a maximum of its activity, since no covalent binding to the polymer matrix is required.

In a preferred embodiment it was found that the best results were obtained by using an aqueous polyurethane dispersion as said aqueous dispersion of a polymer.

The above embodiment was found to be especially useful when said polypeptide is an enzyme such as glucose oxidase, catalase, or lactase.

The permeability modifying agent mentioned above is incorporated into the polymer matrix in order to control the permeability of the end product for small hydrophilic molecules, and it was found that use of various charged high molecular substances was successful.

Especially, when using a membrane of the invention for producing enzyme sensors, it is possible to control the permeability of various interfering substances by the addition of compounds such as heparine, alginic acid, or albumine.

Negatively charged substances such as heparine and alginic acid are especially useful for reducing the

permeability of interfering anions, whereas neutral substances such as albumine are useful in controlling the permeability of the substance to be determined.

Although it is not necessary, it was found practical for
5 obtaining membranes of a reasonable flexibility and avoid formation of cracks to add a plasticizing agent to said aqueous dispersion in step a).

The plasticizer used may be any of the plasticizers normally used within the polymer industry, but for the
10 purposes of this invention preferably dibutyl phthalate was used.

It was also found that the addition of a coalescence agent to said aqueous dispersion in step a) was of great use for the fusion (coalescence) of the individual particles in
15 the dispersion.

Any suitable high boiling solvent well known to the practitioner may be used, among such solvents ethyl carbitol, butyl carbitol, or N-methyl-2-pyrrolidone may be mentioned.

20 It was surprisingly found that it was possible to maintain the activity of enzymes immobilized within the polymeric matrix even when subjecting said dried shaped bodies to a mild heat treatment at a temperature of from about 40 C to about 80 C for a period of time of from about
25 30 minutes to about 30 hours.

Without being bound to any specific theory it is believed that said heat treatment improves the process by evaporating organics, such as the coalescence agent, and provide a smooth surface free of cracks or pores in the
30 finished product.

It is also believed that this heat treatment has made it possible in contrast to other workers in the field to provide for a very high yield of functional membranes for use in biosensors.

35 Also, in the case of the production of a multi-layered membrane, it is believed that said heat treatment provides

for a partial or full fusion of each separate layer to neighbouring layers.

In the preferred embodiment of the invention the subject membrane is advantageously applied by dip-coating, viz. the
5 detecting surface of a biosensor, onto which the membrane must be applied, is carefully dipped into the above dispersion prior to the drying and optional heating steps.

The membrane may of course also be applied to a surface by spraying or any other conventional method of applying a
10 coating (brushing, rolling, etc.).

By the invention there is also provided for a shaped body comprising a polypeptide, such as an enzyme immobilized in a polymer matrix, such as polyurethane.

Said shaped body may have any convenient form, but
15 preferred are small beads and membranes, especially membranes.

In a further aspect the invention also provides for an electrochemical sensor comprising an anode, a cathode, and a detection surface, wherein said detection surface is coated
20 with one or more layers of a membrane produced and applied by any of the above mentioned methods.

In a preferred embodiment of such a sensor said layer(s) are provided with one or more - outer - membrane of similar composition as said layers except for being devoid
25 of any immobilized polypeptide or enzyme.

As a further feature by the invention it was found that sensors according to the invention could be sterilized easily by using a thiomersal containing test buffer during the conditioning period for the sensor.

30

EXAMPLE 1

MONO-LAYER NEEDLE ELECTRODE

a) Physical construction of the electrode.

The electrode is shown in a longitudinal cross section
35 in fig. 1, and is generally designated 1. It comprises a core platinum anode 2 coated with an insulating lacquer

3, the anode 2 is situated inside a stainless steel reference cathode 4 which is insulated from the anode 2 by the lacquer 3 and a layer of epoxy resin 5. At one end, the tip, the electrode 1 has a detection surface 6, which is in an acute angle to the general direction of the electrode 1. At the other end, the base, the electrode 2 is provided with terminals 7 and 8 for the anode 2 and cathode 4, respectively. The terminals 7 and 8 are soldered to leads 9 and 10, respectively, which are connected to instrumentation used when performing measurements.

The electrode assembly is produced by inserting the commercially available lacquer insulated platinum wire 2, 3 with a diameter of 0.16 mm including the lacquer coating, into a stainless steel tube 4 with an outer diameter of 0.46 mm, and finally the anode 2 is fixed in a non-conductive position in relation to the cathode 4 by embedding it in epoxy resin 5 inside the tube 4.

Working and reference electrodes 2 and 4 are subsequently at 7 and 8 soldered to the leads 9 and 10 of a low-loss or sub-miniature coaxial cable. All soldered connections are then embedded into epoxy resin.

Finally the electrode tip is ground to an angle of approximately 15° and polished by honing the tip on a honing stone, whereby a smooth detection surface 6 level with the electrode tip, and an easy insertion of the electrode for in vivo measurements is obtained.

b) Production and application of an enzyme immobilized in a membrane.

Mono-layer membranes were produced according to the following procedure:

The electrode assembly as produced by a) above is defatted and cleansed by piercing multiply folded lens tissue soaked in Ethyl Cellosolve^R 4 to 6 times, whereafter a potential of 650 mV is applied to the platinum anode 2,

and the detection surface 6 dip-coated by dipping into the aqueous polymer dispersion of the invention to produce a coating A. The coating A is dried at room temperature while the detection surface 6 is kept in a horizontal position
5 for a time sufficient for the current to decrease to at least 0.1 nA. Subsequent to this drying the coating A is subjected to a heat treatment at 45 C for 24 hours.

Prior to use or testing the dry sensor 1 must be conditioned by immersing it into a buffer solution of for
10 example the following composition

TEST BUFFER

	Na ₂ HPO ₄ 2 H ₂ O	5.77 g
	NaH ₂ PO ₄ H ₂ O	1.05 g
15	Human albumine	1.00 g
	Thiomersal	0.24 g
	NaCl	6.00 g
	Demineralized water ad	1000 ml
	pH between 7.3 and 7.5	

20

while a voltage of 650 mV is applied to the anode 2.

The sensor is deemed usable when a stable signal is generated. This typically is obtained within a period of from 5 to 24 hours. If it is impossible to obtain a stable
25 signal within the specified period, the sensor is discarded.

As indicated above this conditioning also serves to sterilize the sensor through the activity of the thiomersal in the buffer solution. Experiments have shown that the thiomersal has no effect on the electrochemical
30 characteristics of the biosensor.

c) Testing of sensors.

Mono-layer electrodes produced as described above and with membrane compositions as indicated below were tested in
35 an experimental set-up as outlined in fig. 2.

In fig. 2 a glucose containing sample buffer 11 is contained in a beaker 12. A sensor 1 is placed in a test stand 13 in a position where the detection surface 6 is immersed into the sample buffer, and the lead 9 from the anode 2 is connected to the positive terminal on a stabilized power supply 14 applying a voltage of 650 mV. The cathode 4 is connected to a current monitoring device 15, such as an amperometer, a recorder, or similar equipment through a cable 10. The monitor 15 and the power supply 14 are in turn connected through a cable 16.

In the actual set-up for the testing of the sensors of the invention the monitor 15 used was a Keithley picoamperometer Model 485, and the power supply 14 was an 1.5 V dry cell connected to a voltage divider.

Materials.

- Polyurethane dispersion. A stock dispersion produced by incorporating 16 weight% dibutylphthalate (Merck-Schuchardt) in 84 weight% commercial polyurethane dispersion (NeoRez^R R-974 from Polyvinyl Chemie Holland bv, Waalwijk, Holland), and adding an equal amount by weight of water.
- Ethyl carbitol (Merck),
- Sodium alginate. A stock solution of 2% (Sigma) in water was used.
- Sodium heparine. A stock solution of 2% (NOVO INDUSTRIAL A/S) in water was used.
- Glucose oxidase. A stock solution of 4.78 mg/ml (240 U pr. mg) (Serva).

Tests.

Tests were performed with mono-layer membranes produced from mixtures of the compositions indicated in Table I.

TABLE I

	Component	Amount	
		mix 1	mix 2
5	Polyurethane dispersion	108 mg	
	Polyurethane, pure NeoRez ^R R-970		100 mg
	Ethyl Carbitol		110 mg
	Butyl Carbitol		
	10 weight% in water (demin.)	921 mg	
10	Sodium alginate		100 mg
	Glucose oxidase	150	150
	Ferrocene aldehyde		5 mg
	Water (demin.)		635 mg

15 Two series of sensors were produced, one using mix 1 and designated sensor 1, was dried at room temperature for 24 hours, and the other using mix 2 and designated sensor 2, was heat treated at 60 C for one hour.

From Table I it is seen that incorporation of ferrocene
20 aldehyde in the polymer matrix was possible.

These mono-layer sensors were produced less effectively than the multi-layer sensors mentioned below in Example 2, since some had to be discarded due to instability.

Subsequent to conditioning the sensors were tested in
25 the test buffer, to which aliquots of glucose was added to obtain concentrations of glucose in the buffer. The results from a testing of the above two mono-layer sensors are indicated in table II below.

30 Table II

	sensor 1	sensor 2
Sensitivity(current) at 12 mM glucose	5.9 nA	
Sensitivity(current) at 10 mM glucose		2.4 nA
Residual current at 0 mM glucose	<0.1 nA	<0.1 nA
35 Linear to at least (mM glucose)	20	40
Correlation coefficient (R)	0.999	0.999

Sensor 2 was also tested in vivo in a pig. The sensor was introduced in an ear vene through a Venflon^R catheter. Blood samples were taken at intervals from the other ear and
5 the glucose concentration measured by standard analysis in order to determine the correlation between the observed current in the sensor and the blood glucose content.

The result of this test was that the current in the sensor varied from 3 nA to 12 nA while the control
10 measurements varied from 2.4 mM glucose to 25 mM glucose. This shows that usually it is necessary to calibrate the sensor in situ prior to trusting the sensor measurements.

EXAMPLE 2

15 MULTI-LAYER NEEDLE ELECTRODE

a) Physical construction of the electrode.

As shown in fig. 3 the physical construction of multi-layer electrodes was identical to the mono-layer type except for the number of layers in the membrane.

20

b) Production and application of an enzyme immobilized in a membrane.

The multi-layer membranes were produced according to the following procedure:

25 Each layer A, B, C, D shown in fig. 3 is applied as for the mono-layer electrode except for the heat treatment which is only performed when the desired number of layers A, B, C, D has been applied.

The composition of the layers is usually identical
30 except for one or two outermost layers which are devoid of enzyme.

Again the sensor must be conditioned prior to testing and/or use in the test buffer.

35 Tests

For this example two new mixtures were used, one without glucose oxidase for the outer layers in the membrane, and one containing glucose oxidase for the inner layers.

- 5 The compositions of the two mixtures are indicated in Table III below.

Table III

	Component	Volume%	
		mix 3	mix 4
10	polyurethane dispersion	40	40
	Water (demin.)	40	25
	ethyl carbitol	10	10
	sodium alginate	10	10
	glucose oxidase		15

15

From these mixtures one series of sensors was made comprising two inner layers from mix 4 and two outer layers from mix 3.

- 20 The layers were applied in a manner similar to that described in example 1, except that each individual layer was allowed to dry at room temperature for approximately 5 minutes prior to application of the next coating, and finally the four-layer membrane was heat treated at 65 C for 45 minutes.

- 25 The structure of this four-layer needle sensor is shown in fig. 3, where it is seen that in all other respects than the membrane A, B, C, D the structure is identical to the structure of the mono-layer sensor shown in fig. 1.

- 30 Compared to the production of the mono-layer electrode the multi-layer electrodes proved more successful in respect of "yield" of usable electrodes.

Sensors from this production were similarly to example 1 tested for their response after conditioning. The results from this testing is shown in Table IV below

35

Table IV

	Sensitivity at 5 mM glucose	1.5 nA +/-20%
	Residual current at 0 mM glucose	<0.1 nA
	Linear to at least (mM glucose)	20*
5	Correlation coefficient (R)	0.999
	*The sensors were tested at 0, 2, 10, 15, and 20 mM glucose at 23 C.	

Long term stability

10 A number of the sensors from this batch were tested for "long-term" stability by placing them in the test buffer containing 5 mM glucose at 23 C and monitoring the result for at least 80 hours.

By this test it was found that the current varied less
15 than 0.5% during this period.

Permeability control

In order to determine the dependency of the response from variations in alginate content a batch of sensors were
20 produced wherein the membrane comprise two layers from mix 4 and two layers from mix 3 modified by substituting 100 sodium alginate solution with 10 sodium alginate solution plus 90 water.

The results from this test are shown in Table V beow.
25

Table V

	Sensitivity at 5 mM glucose	0.5 nA +/-20%
	Residual current at 0 mM glucose	<0.1 nA
	Linearity to at least (mM glucose)	7*
30	Correlation coefficient (R)	0.999
	*The sensors were tested at 0, 5, and 7 mM glucose.	

From Table V it is clearly seen that by reducing the alginate content in the membrane it was possible to reduce
35 the sensitivity of the sensor by controlling the glucose permeability of the membrane.

CLAIMS

- 5 1. A method for immobilizing a polypeptide such as an enzyme in a polymeric matrix comprising the following steps:
a) adding said polypeptide and a permeability modifying agent to an aqueous dispersion of a polymer to produce an aqueous dispersion wherein said polypeptide is dissolved;
10 b) forming said dispersion into one or more bodies of a desired shape;
c) leaving said shaped body for a period of time from about 5 minutes to about 200 minutes at room temperature for drying;
- 15 2. A method according to claim 1, wherein said aqueous dispersion of a polymer is an aqueous polyurethane dispersion.
3. A method according to claim 1 or 2, wherein said polypeptide is an enzyme, preferably a glucose oxidase,
20 catalase, or lactase.
4. A method according to any of the claims 1 to 3, wherein said permeability modifying agent is a high molecular hydrophilic substance, preferably heparin, alginic acid, or albumine.
- 25 5. A method according to any of the claims 1 to 4, wherein a plasticizing agent is added to said aqueous dispersion in step a), preferably dibutyl phthalate.
6. A method according to any of the claims 1 to 5, wherein a coalescence agent is added to said aqueous
30 dispersion in step a), preferably a suitable high boiling solvent, and most preferred ethyl carbitol, butyl carbitol, or N-methyl-2-pyrrolidone.
7. A method according to any of the claims 1 to 6, wherein subsequent to step c) a further step d) is performed
35 by

d) subjecting said dried shaped bodies to a heat treatment at a temperature of from about 40 C to about 80 C for a period of time of from about 30 minutes to about 30 hours.

8. A method for immobilizing an enzyme in a polymeric matrix comprising the following steps:

- a) adding said enzyme, a permeability modifying agent, a coalescence agent, and a plasticizing agent to an aqueous dispersion of polyurethane polymer to produce an aqueous dispersion wherein said enzyme is dissolved;
- b) forming said dispersion into one or more bodies of a desired shape;
- c) leaving said shaped bodies for a period of time from about 5 minutes to about 200 minutes at room temperature for drying;
- d) subjecting said dried shaped bodies to a heat treatment at a temperature of from about 40 C to about 80 C for a period of time of from about 30 minutes to about 30 hours.

9. A method according to claim 8, wherein said enzyme is chosen from the group comprising glucose oxidase, catalase, and lactase, preferably glucose oxidase.

10. A method according to claim 8 or 9, wherein said permeability modifying agent is chosen from the group comprising heparine, alginic acid, and albumine.

11. A method according to any of the claims 8 to 10, wherein said coalescence agent is chosen from the group comprising ethyl carbitol, butyl carbitol, and N-methyl-2-pyrrolidone.

12. A method according to any of the claims 8 to 11, wherein the plasticizing agent is dibutyl phthalate.

13. A method according to any of the claims 1 to 12, wherein said aqueous dispersion in step b) is formed to a membrane.

14. A method according to claim 13, wherein said membrane is produced by dip-coating.

15. A method according to claim 13, wherein said membrane is produced by spraying.

16. A shaped body produced by a method according to any of the claims 1 to 15.

17. A membrane produced by a method according to any of the claims 1 to 15.

5 18. A polymeric membrane wherein a polypeptide such as an enzyme has been immobilized by physical entrapment in a polymeric matrix.

19. A membrane according to claim 18 further comprising a permeability modifying agent.

10 20. A membrane according to claim 19, wherein said enzyme is chosen from the group comprising glucose oxidase, catalase, and lactase, preferably glucose oxidase.

21. A membrane according to claim 19 or 20, wherein said permeability modifying agent is chosen from the group
15 comprising heparine, alginic acid, and albumine.

22. An electrochemical sensor comprising an anode, a cathode, and a detection surface, wherein said detection surface is coated with one or more layers of a membrane according to any of the claims 17 to 21.

20 23. An electrochemical sensor according to claim 22, wherein said layer(s) are provided with one or more further - outer - layer(s) of the same composition as said layers except for being devoid of any immobilized polypeptide or enzyme.

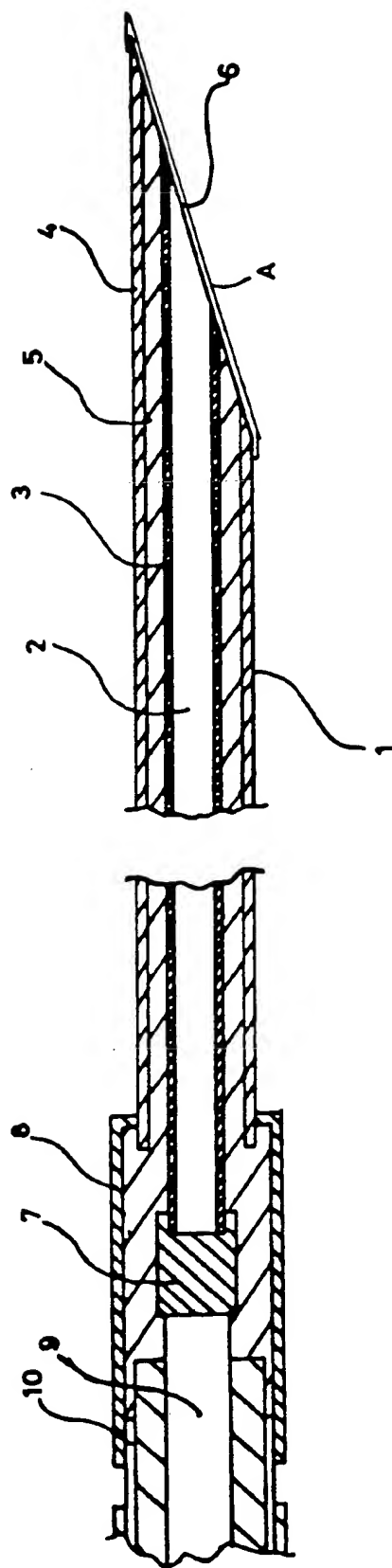


Fig.1

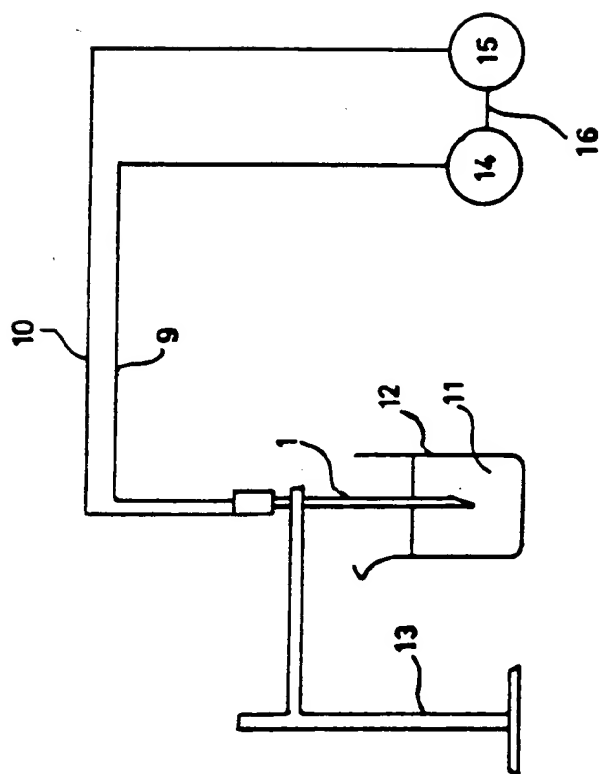


Fig.2

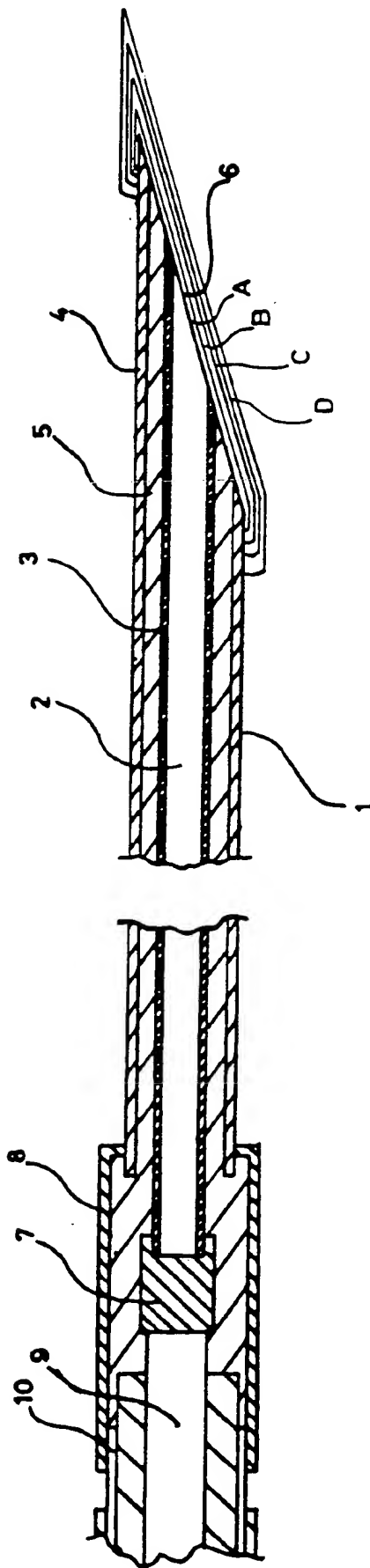


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK89/00019

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC 4 C 12 N 11/08, /04, C 12 M 1/40						
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ?</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC 4</td> <td style="padding: 5px;">C 12 N; C 12 M; C 12 Q</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div> <p style="padding: 5px;">SE, NO, DK, FI classes as above. Data base search CA,WPI,WPIL.</p>			Classification System	Classification Symbols	IPC 4	C 12 N; C 12 M; C 12 Q
Classification System	Classification Symbols					
IPC 4	C 12 N; C 12 M; C 12 Q					
III. DOCUMENTS CONSIDERED TO BE RELEVANT †						
Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages †‡	Relevant to Claim No. ‡‡				
X Y	DE, A1, 3 617 875 (HITACHI PLANT ENGINEERING & CONSTRUCTION CO., LTD) 8 January 1987 See claim & JP, 62003787 US, 4791061 JP, 62061583	1-5,8-12,16 13-23				
Y	US, A, 4 094 744 (FRANK JOSEPH HARTDEGEN et al) 13 June 1978 See example 1 & BE, 860941 NL, 7712661 FR, 2371470 DE, 2750803 GB, 1542009 JP, 53066491 AT, 362494 CA, 1106300 CA, 1108072 CH, 630931 <div style="text-align: right; padding-top: 10px;">.../...</div>	1-3,7				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: †‡</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search 1989-05-15	Date of Mailing of this International Search Report 1989-05-21					
International Searching Authority Swedish Patent Office	Signature of Authorized Officer Yvonne Siösteen					

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0 222 289 (BAYER AG) 20 May 1987 & DE, 3540333 JP, 62118889	1-3,7-9
Y	EP, A2, 0 280 212 (BOEHRINGER MANNHEIM GMBH) 31 August 1988 DE, 3705687 JP, 63226283	1-3,7-9
Y	US, A, 4 407 957 (FRANKLIN LIM) 4 October 1983 See column 1, line 39-41, column 7, line 6-12 & BE, 892477 GB, 2094750 FR, 2501528 DE, 3209045 SE, 8201557 JP, 57197031 CA, 1184518 CH, 651579 SE, 454181	4,10,13-21
Y	Chemical Abstracts, Vol 103 (1985) abstract No 200839n, Polym.Mater. Sci.Eng. 1985, 53, 423-5 (Eng)	4,10,21
Y	EP, A2, 0 216 577 (IMPERIAL CHEMICAL INDUSTRIES PLC) 1 April 1987 See claim 3 and example 1 page 6 line 16-17 & JP, 62067442 CA, 1244085	4,10,13-17, 21-23